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Pretreatment of Human Epidermal Keratinocytes In Vitro with Ethacrynic Acid Reduces Sulfur Mustard Cytotoxicity

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Sulfur mustard (SM) is a potent alkylating agent, profoundly cytotoxic, and a powerful vesicant. SM reacts quite extensively with glutathione (GSH) and forms GSH conjugates, which are presumably excreted through the mercapturic acid pathway in mammals. It is unknown whether any enzymes, such as the glutathione-S-transferases (GST), are involved in this detoxification of SM by the formation of conjugates. A prototypic inhibitor (ethacrynic acid, EAA) and a prototypic inducer (Oltipraz, OLT) of GSH-S-transferase, have been used as pretreatment compounds in human epidermal keratinocytes (HEK) to investigate the effect of enzyme levels on cytotoxicity following SM challenge from 50 μM to 300 μM. Pretreatment of HEK for 24 h with EAA doubled survival against 200 μM SM (36% viability in non-pretreated cells vs. 81% in EAA-pretreated cells) and quadrupled survival (17% viability in non-pretreated controls vs. 71% in EAA-pretreated cells), while OLT pretreatment had no effect on cytotoxicity at either SM dose. The role of GST in SM cytotoxicity could not be tested because of the lack of an effect on modulation of GST activities by these 2 drugs. Cellular levels of GSH were increased 250–300% over control values using EAA pretreatment, while OLT pretreatment did not lead to any increase in GSH. Pretreatment of HEK with buthionine sulfoximine (BSO), a known depleter of glutathione levels, reduced glutathione levels and increased cytotoxicity. This large increase in GSH appears to be solely responsible for the enhanced survivability of EAA-pretreated HEK.

Keywords Cytotoxicity, Ethacrynic Acid, Pretreatment, Sulfur Mustard

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Sulfur mustard (SM) is mutagenic, carcinogenic, radiomimetic, cytotoxic, and reacts with a variety of macromolecules that appear to be contributory to its biochemical mechanism of action. Additionally, this compound is best known for its vesicant (blister-forming) ability as a chemical warfare agent that has been used over the past century in numerous battlefield conflicts. It affects ocular, respiratory, and cutaneous human systems (Papirmeister et al. 1991), and there is no medical countermeasure presently available that can interfere with its pathology. Despite research efforts over the last 80 years, understanding of the processes that produce these varied effects remains elusive.

SM has a very short half-life under aqueous conditions and is a lipophilic compound that appears to penetrate the cell by diffusion. When SM forms the reactive cyclic ethylene sulfonium ion, there is alkylation of many intracellular macromolecules. The tripeptide glutathione (GSH) is also a cellular target of this agent and has been found to react extensively with SM (Davison et al. 1961; Roberts and Warwick 1963; Black et al. 1992), forming a conjugate. The conjugate goes through a number of steps before it is cleaved by β-lyase and excreted as a mercapturic acid and other cysteine conjugates (Black et al. 1992). Management of intracellular levels of GSH may decrease SM cytotoxicity, but this approach has had only limited success (Gross et al. 1997; Gross et al. 1993) since only modest increases of intracellular GSH were formed.

Attempts to modify SM cytotoxicity by manipulation of glutathione-S-transferase (GST) and other detoxification enzymes have not been studied in detail. The effect of these enzymes on the conjugation of the electrophile, SM with the nucleophile, GSH has not yet been assessed. Pretreatment of HEK with EAA, a potent diuretic and a prototypic inhibitor of GST,

may exacerbate the effects on SM-induced cytotoxicity by decreasing formation of the conjugate. Conversely, pretreatment of human epidermal keratinocytes with OLT, a prototypic inducer of GST, may decrease the effects on SM-induced cytotoxicity by increasing the formation of the conjugate. These two compounds with differing effects on GST levels will be investigated to determine their role in SM cytotoxicity to HEK.

In addition, EAA also appears to have the capability of both reducing (Ploemen et al. 1990) and increasing GSH levels in cells (Chen and Waxman 1994) when incubated for longer times. A positive control, buthionine sulfoximine, for reduction of GSH levels by the inhibition of its synthesis will also be assessed. EAA or OLT may also affect the cytotoxicity of SM to HEK by virtue of influencing intracellular GSH levels.

MATERIALS AND METHODS

Materials

Normal Human Epidermal Keratinocytes (HEK), Keratinocyte Growth Media (KGM), and Trypsin-EDTA Reagent Packs were purchased from Cambrex Corporation (Walkersville, MD). Tissue culture vessels were purchased from Corning Corporation (Corning, NY) or Falcon Corporation (Newark, NJ). Cell Titer 96® AQueous Non-radioactive Cell Proliferation kits from Promega (Madison, WI) allow the quantitation of living cells by measuring the conversion of the unique tetrazolium compound (3-(4,5-dimethyl thiazol-2-yl)-5-(carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt; MTS) to a yellowish water-soluble formazan in the presence of an electron coupling reagent, phenazine methosulfate (PMS), by dehydrogenase enzymes. The amount of formazan formed is then directly proportional to the number of living cells in culture. Ethacrynic acid (EAA), buthionine sulfoximine (BSO), and other laboratory chemicals were purchased from Sigma Chemical Company (St. Louis, MO). Oltipraz (OLT) was obtained from Walter Reed Army Institute of Research. Sulfur mustard (CAS registry # 505-60-2, 96.8% pure) was obtained from Edgewood Chemical Biological Center, Aberdeen Proving Ground, MD, USA.

Cell Culture

HEK were purchased as second passage cells and cultured in KGM in T-75 flasks in a 5% CO_2 incubator at 37°C. They were subsequently subcultured into 24-well or 96-well plates at the appropriate density. Cells were grown to estimated confluencies of 60–90% prior to experimentation.

Pretreatment of HEK with EAA, OLT, or BSO

HEK subcultured in 24- or 96-well plates at the appropriate confluencies were exposed to various concentrations of EAA (3, 5, and 10 μg/well), OLT (10, 50, and 100 μg/well), or BSO (2, 22, and 220 μg/well) for 0, 2, 4, 8, 24, and 48 h. Media was then removed by aspiration, and the plates were washed twice with appropriate volumes of phosphate buffered saline, pH 7.5. Cells from each pretreatment drug were then divided into 2 groups: (1) frozen on dry ice and stored at 80°C for later analysis of GSH or GST, (2) or fresh KGM was added to the plates for further experimentation with SM.

GSH Analysis of HEK

The 24-well plates of HEK that had been pretreated with EAA, OLT or BSO for 0, 2, 4, 8, 24, or 48 h, washed, and then and stored at −80°C were thawed. Aliquots of 300 μL of 5% sulfosalicylic acid were added, and the plates were extracted overnight at 4°C. The plates were centrifuged at 500 g for 15 min, and the supernatant liquid was withdrawn and placed in conical tubes. Aliquots of 15 μL supernatant fluid were then analyzed for total GSH (GSH + GSSG) by the GSH reductase cycling assay (Griffith 1980).

GST Analysis of HEK

Frozen HEK were thawed by the addition of 300 μL of 0.1 M KPO_4 buffer, pH 6.5 and immediately lysed by sonication in a Misonix plate sonicator at 4°C for 4–30 sec bursts using 1-min cooling intervals. The sonicate (50 μL) was transferred to a fresh plate and analyzed for GST (Gercia Alfonso et al. 1998). Enzymatic activities were expressed as % of control activity.

Exposure of Cells to SM

A stock solution of 4 mM SM was diluted into KGM and added to the appropriate cell culture plates in a chemical surety hood to yield final SM concentrations of 10, 50, 100, 200, 300, and 500 μM. After one hour at ambient temperature to allow reaction and hydrolysis of agent, the tissue culture plates were transferred to a 37°C incubator under a humidified 5% CO_2 atmosphere for post-exposure incubation. Removal of SM is unnecessary since it is hydrolyzed to innocuous products.

Assessment of HEK Cytotoxicity in 24-Well Plates

Cell viability in 24-well plates was determined by using the MTS-PMS assay. At 24 h after SM exposure, the plates were removed from the incubator and 500 μL of media was removed and discarded. Fifty μL of the MTS-PMS solution was added to each well containing 500 μL of KGM. The plates were incubated at 37°C in a 5% CO_2 incubator for an additional 4 h and read at 490 nm with a Molecular Devices Spectramax microplate scanning spectrophotometer. Blank values were subtracted from the data and viability at each dose was expressed as percent of control viability as shown below:

$$\frac{[\text{Absorbance of sample}]}{[\text{Absorbance of control}]} \times 100$$

Assessment of HEK Cytotoxicity in 96-Well Plates

Cell viability in 96-well plates was also determined using MTS-PMS as above. At 24 h after SM exposure, the plates were removed from the incubator, and 20 μL of the MTS-PMS solution was added to each well containing 100 μL of KGM. The plates were incubated at 37°C in a 5% CO_2 incubator for an additional 4 h and read at 490 nm with a Molecular Devices

Spectramax microplate scanning spectrophotometer. Blank values were subtracted and viability was calculated as % of control viability as defined above for 24-well plates.

Statistical Analysis

Data was analyzed by ANOVA and showed a significant difference from controls when $p < 0.05$ (*). Post testing was performed using the Tukey-Kramer Multiple Comparisons Test.

RESULTS

SM toxicity to HEK in 96-well plates was investigated by exposing cells to various concentrations of SM and incubating for 24 h in a 5% CO_2 incubator at 37°C. Cytotoxicity was measured by the MTS-PMS assay as described in Methods. In Figure 1, cytotoxicity was observed beginning at 50 μM SM, increased at 100 and 200 μM, and appeared to reach a maximum at 300 μM SM. Exposure to 500 μM SM was no different statistically ($p > 0.05$) than to 300 μM, so subsequent experimentation used 300 μM as the highest dose.

EAA and OLT were chosen as prototypic compounds to study their different effects on GSTs (GST) in HEK. EAA should be effective at inhibiting GST while OLT should induce GST in HEK. Figure 2 showed no significant differences ($p > 0.05$) between the effects of the two compounds on GST activities at the lower concentrations. As a result of the lack of an effect by these 2 drugs on GST activities, the role of GST in SM cytotoxicity could not be tested. At the highest concentration of 10 μg EAA /well, there was a significant inhibition of GST activity ($p < 0.05$), but the morphology of HEK at this concentration appeared abnormal with altered shapes and rounding of cells (data not shown) so this EAA concentration was not used. There was an increase of intracellular GSH over a 24- to 48-h timespan in cells pretreated with 3 and 5 μg EAA that did not occur in OLT-pretreated cells (Fig. 3, Panels A and B). Intracellular GSH levels with EAA pretreatment were 2–3 times the levels seen with non-treated cells over the same time period. When the pretreatment level of EAA reached 5 μg/well, GSH levels appeared the greatest but dropped lower than that of control cells at EAA levels of 10 μg/well. OLT pretreatment did not cause any significant differences ($p > 0.05$) in GSH levels between control and OLT-pretreated cells. No increase of intracellular GSH appeared to occur over a 24- to 48-h period (Fig. 3, Panel B) as shown with EAA. BSO pretreatment showed the expected loss of intracellular GSH in HEK to less than 10% within 24 h (Fig. 3, Panel C).

The effect of EAA, OLT, and BSO pretreatment on SM cytotoxicity was then investigated as shown in Figure 4. At 100 μM SM, there appeared to be a slight increase in viability with EAA-pretreated HEK, but this observation was not statistically valid. At 200 μM SM, there was a doubling in survival (viability of 36% in non-pretreated controls vs. 81% in EAA-pretreated cells). Using a 300 μM SM challenge, the protective effect of EAA quadrupled survival (viability of 17% in non-pretreated controls vs. 71% in EAA-pretreated cells).

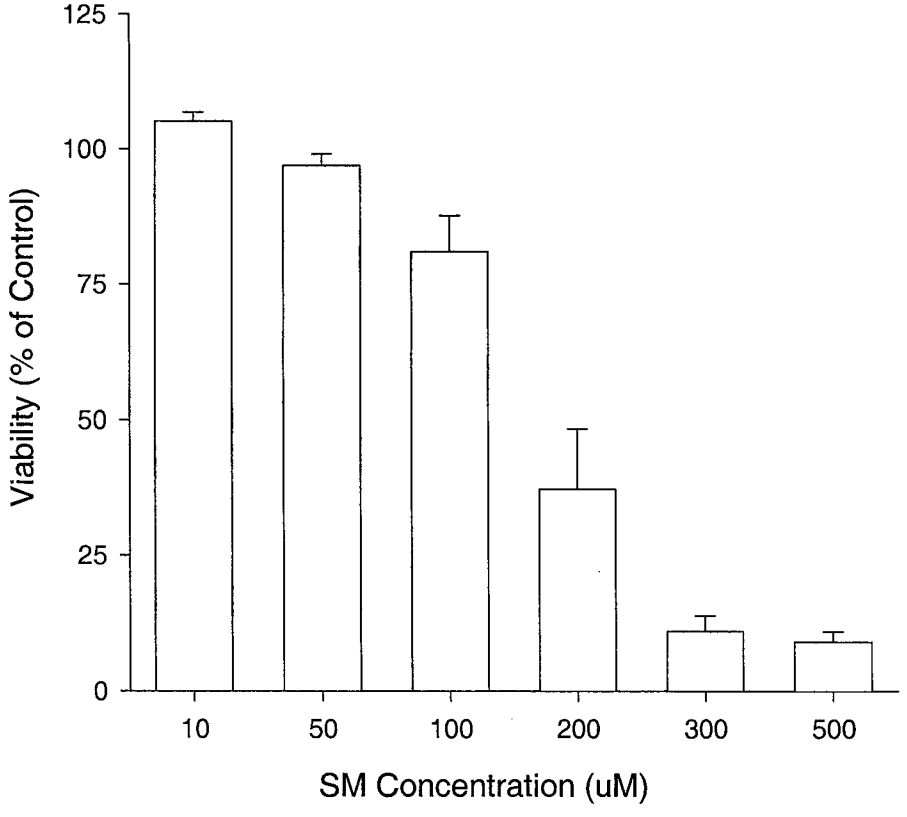

FIG. 1. Effect of SM on viability of HEK. Each bar represents the mean % of control viability ± SEM of 3 separate experiments. Data were analyzed by ANOVA; level of significance was $p < 0.001$ (*) compared with untreated controls.

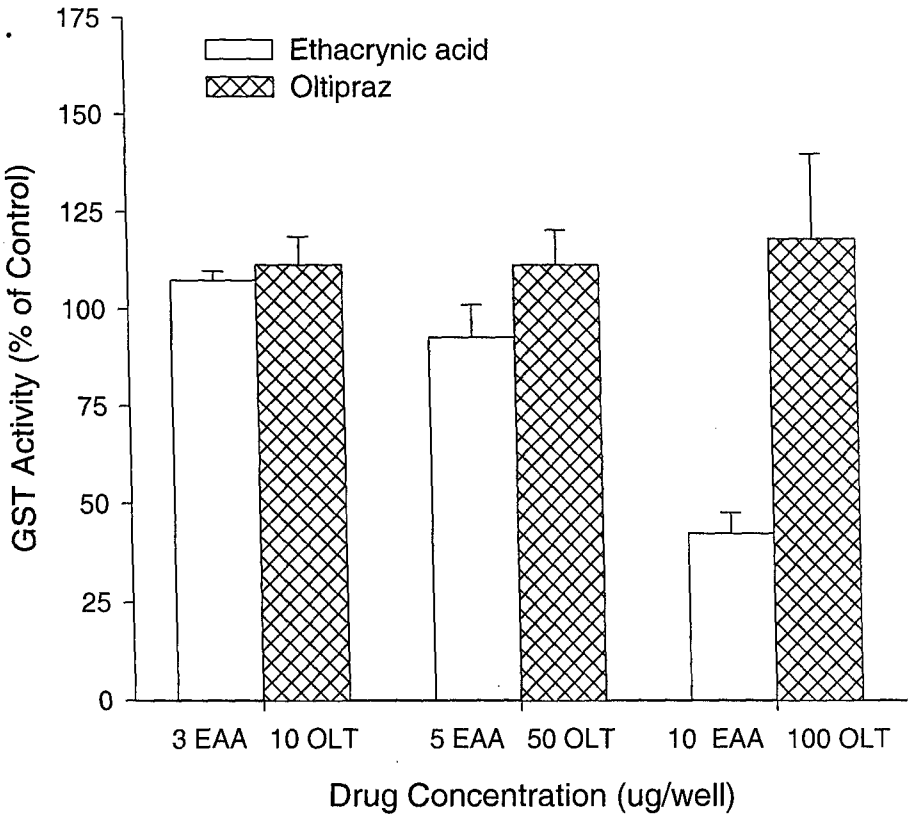

FIG. 2. Effect of prototypic drugs on GST levels in HEK. Each bar represents the mean ± SEM of 3 separate experiments. Data were analyzed by ANOVA; level of significance was $p < 0.01$ comparing drugs at 3 different levels.

OLT pretreatment had no protective effect against cytotoxicity at all of the SM doses studied. There were no statistical differences in viability seen between the non-pretreated HEK and the OLT-pretreated cells.

As expected, BSO-pretreatment showed an increase in cytotoxicity at all concentrations of SM. At 100 μM SM, there was a viability of 47% in BSO-pretreated cells vs. 73% in non-pretreated cells. At 200 μM SM, cytotoxicity increased (viability of 12% in BSO-pretreated cells vs. 36% in non-pretreated controls). At 300 μM SM, BSO-pretreatment showed a further exacerbation of cytotoxicity (viability of 5% in BSO-pretreated cells vs. 17% in non-pretreated controls).

DISCUSSION

In addition to hydrolysis and oxidation of SM to relatively innocuous products, one of the most common detoxification reactions of SM occurs by alkylation of the tripeptide, GSH. Moderately high levels of this compound exist within cells and may act as a "scavenger" for intracellular SM. This alkylkation of GSH has been thought to occur spontaneously, but the role of specific enzymes like the GSTs has not been investigated in any detail. These enzymes transfer electrophilic alkylating agents, such as SM, to the nucleophilic GSH and form a conjugate that is ultimately excreted through the mercapturic acid pathway in mammals. The intermediate products formed during this elimination of SM have been putatively identified (Black et al. 1992).

If GSTs are involved during this detoxification, induction of these enzymes may accelerate the formation of the sulfur-mustard-GSH complex. The amount of SM available to alkylate critical biological targets would decrease, and there would be a corresponding decrease in cytotoxicity. Conversely, an inhibition of the transferases may cause a decrease in the postulated complex, thereby increasing the cytotoxic effect of SM.

Because cytotoxicity measurements were selected as the biological marker, an assay was needed that could determine this effect in both 24- and 96-well plates of HEK. The MTS-PMS kit developed by Promega appeared to be satisfactory as an inexpensive and rapid technique to assess cytotoxicity in SM-treated HEK. With this method, a commonly available plate-scanning spectrophotometer can be used to measure hundreds of samples within a few minutes. This procedure has demonstrated reproducibility of dose response curves in HEK following SM exposure without the need for enzymatic dissociation of these adherent cells. By the use of appropriate blanks, dose response curves can be easily and rapidly generated and potential medical countermeasures against SM and other chemical agents can be investigated quickly.

EAA was selected as a prototypic compound to study the effect of GST on SM cytotoxicity since it is a reputed inhibitor of GST. If the GST were involved in the detoxification of the

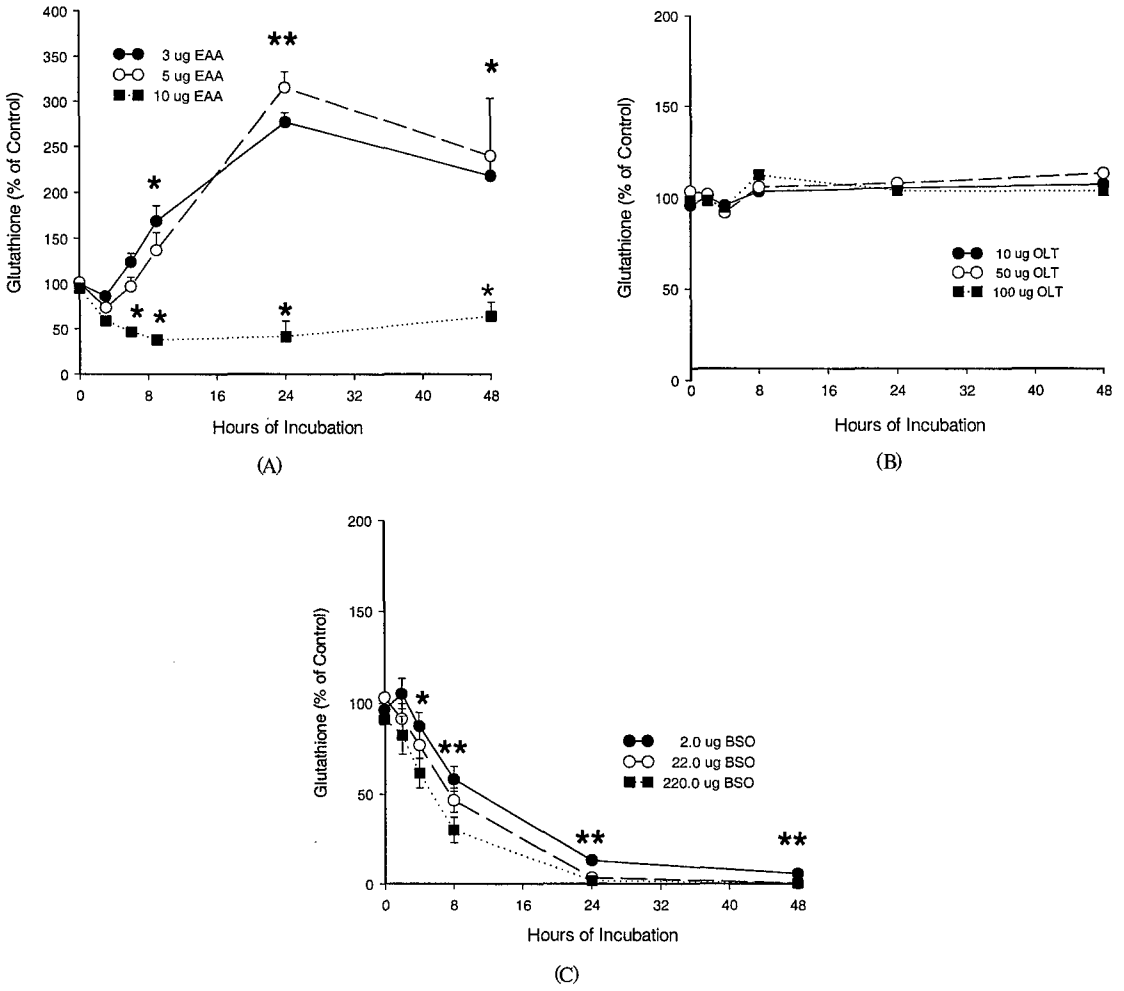

FIG. 3. Effect of various EAA (Panel A), OLT (Panel B), and BSO (Panel C) doses on intracellular GSH levels over 48 hours. Symbols represent mean values ± SEM for 3 experiments with each drug. Data were analyzed by ANOVA and levels of significance were $p < 0.05$ (*), $p < 0.001$ (**) compared with untreated controls.

sulfur mustard by forming conjugates with GSH, the inhibition of this enzyme should exacerbate the cytotoxicity of SM. Very little inhibition of this enzyme is seen except at the highest concentration of EAA at 10 μg/well, which also affected the morphology of the cells, so it was not further investigated. OLT was selected as a prototypic inducer of GST in HEK but did not show the expected increase of GST at any of the concentrations studied. Neither drug was effective in the modification of GST levels in HEK in the current study, and the reason for this discrepancy in HEK is not known.

There appeared to be a large intracellular increase of GSH levels within HEK when they were incubated with EAA. This peak in GSH appeared at 24 h in HEK that were exposed to 5 μg/well and was 2- to 3-fold the GSH levels in control cells. There was no effect on GSH levels in HEK that had been exposed to OLT for comparable times. BSO showed a predictable pattern in depleting GSH levels in HEK similar to studies performed previously on human peripheral lymphocytes. Although these studies (Gross et al. 1997, 1993) on enhancing GSH levels with cysteine-containing prodrugs to protect against SM cytotoxicity had shown marginal protection, GSH levels in these studies were only 25–50% above control levels.

Since GSH levels in EAA treated cells were 2- to 3-fold that of untreated cells, the effect on cytotoxicity was studied in HEK pretreated with EAA levels of 3 μg/well and showed increases in cytoprotection against SM. The EAA pretreatment was ineffective against SM for up to 6 h (data not shown) and may reflect a drug penetration problem or a lag in GSH synthesis. The 24-h pretreatment timepoint showed protection against 200 and 300 μM SM corresponding to the peak in GSH levels. Pretreatment of HEK with OLT at 50 μg/well did not show any protection from SM cytotoxicity, whereas pretreatment with BSO showed an exacerbation of cytotoxicity in accordance with other studies (Gross et al. 1997).

GST level modification by EAA or OLT pretreatment was not observed in this study. EAA pretreatment in HEK showed cytoprotection against SM, OLT showed no effect, and BSO showed enhanced cytotoxicity. The large increase in GSH observed here

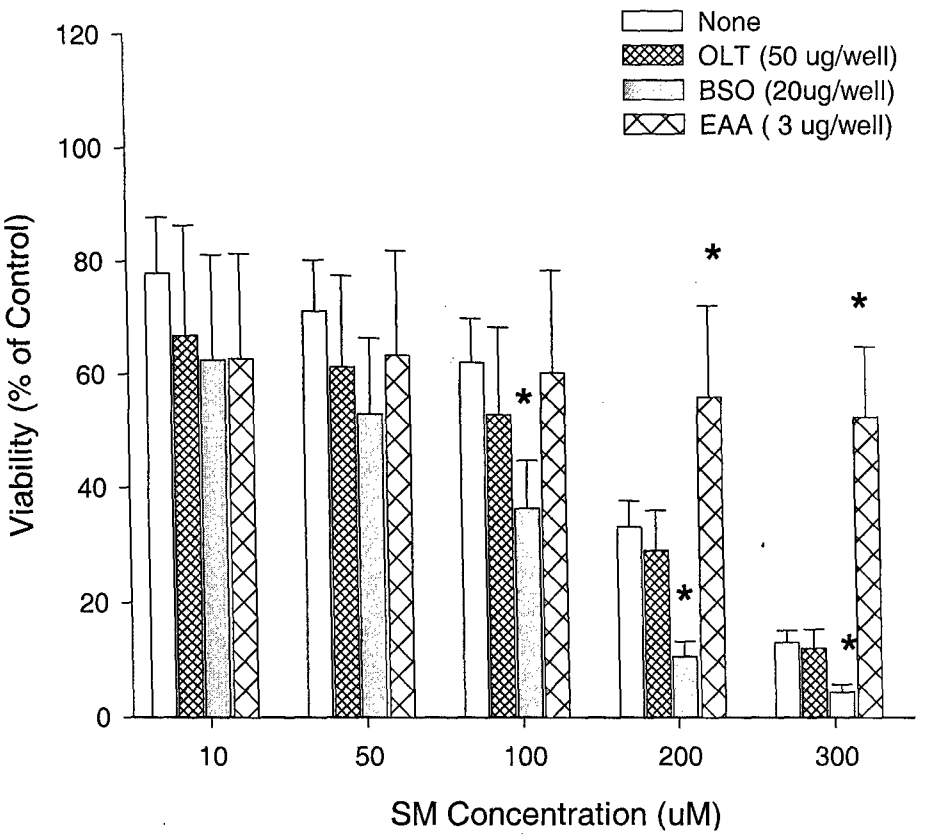

FIG. 4. Effect of drug pretreatment on SM cytotoxicity. Bars indicate mean values ± SEM for 3 separate experiments. Data were analyzed by ANOVA; level of significance was $p < 0.05$ (*) compared with non-pretreated controls.

appeared to be solely responsible for the enhanced survivability of EAA-pretreated HEK against SM.

The opinions or assertions contained herein are the private views of the authors and are not to be construed as official or as reflecting the views of the Department of the Army or the Department of Defense, USA.

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